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(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: SANDAL, Thomas [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). KOFOD, Lene, Venke [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). KAUPPINEN, Markus, Sakari [FI/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). ANDERSEN, Lene, Nonboe [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo allé, DK-2880 Bagsværd (DK).

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(54) Title: AN ENZYME WITH XYLANASE ACTIVITY

(57) Abstract

The present invention relates to an enzyme with xylanase activity, a DNA construct encoding the enzyme with xylanase activity, a method of producing the enzyme, an enzyme preparation comprising said enzyme with xylanase activity, a detergent composition comprising said xylanase, and the use of said enzyme and enzyme preparation for a number of industrial applications.

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TITLE: An enzyme with xylanase activity

FIELD OF INVENTION

The present invention relates to an enzyme with xylanase activity, a DNA construct encoding the enzyme with xylanase activity, a method of producing the enzyme, an enzyme preparation comprising said enzyme with xylanase activity, and the use of said enzyme and enzyme preparation for a number of industrial applications.

BACKGROUND OF THE INVENTION

Xylan, a major component of plant hemicellulose, is a polymer of D-xylose linked by beta-1,4-xylosidic bonds. Xylan can be degraded to xylose and xylo-oligomers by acid or enzymatic hydrolysis. Enzymatic hydrolysis of xylan produces free sugars without the by-products formed with acid (e.g. furans).

Enzymes which are capable of degrading xylan and other plant cell wall polysaccharides are important for the feed and food industry. In the feed industri xylanases are primarily used as feed enhancers and for processing of feed. In the food industry xylanases are primarily used for baking, and in fruit and vegetable processing such as in wheat separation, fruit juice production or wine making, where their ability to catalyse the degradation of the backbone or side chains of the plant cell wall polysaccharide is utilised (Visser et al., in "Xylans and Xylanases", Elsevier Science publisher, 1991.

Other applications for xylanases are enzymatic breakdown of agricultural wastes for production of alcohol fuels, for hydrolysis of pentosans, manufacturing of dissolving pulps yielding cellulose, and bio-bleaching of wood pulp [*Detroym R.W.* In: Organic Chemicals from Biomass, (CRC Press, Boca Raton, FL, 1981) 19-41.; *Paice, M.G., and L. Jurasek.*, J. Wood Chem. Technol. 4: 187-198.; *Pommier, J.C., J.L. Fuentes, G. Goma.*,

Tappi Journal (1989): 187-191.; *Senior, D.J., et al.*, Biotechnol. Letters 10 (1988):907-912].

WO 92/17573 discloses a substantially pure xylanase derived from the fungal species *H. insolens* and recombinant DNA encoding said xylanase. The xylanase is stated to be useful as a baking agent, a feed additive, and in the preparation of paper and pulp.

WO 92/01793 discloses a xylanase derived from the fungal species *Aspergillus tubigensis*. It is mentioned, but not shown that related xylanases may be derived from other filamentous fungi, examples of which are *Aspergillus*, *Disporotrichum*, *Penicillium*, *Neurospora*, *Fusarium* and *Trichoderma*. The xylanases are stated to be useful in the preparation of bread or animal feed, in breewing and in reducing viscosity or improving filterability of cereal starch.

Shei et al.(Biotech. and Bioeng. vol XXVII 553-538, 1985), and Fournier et al.(Biotech. and Bioeng. vol XXVII 539-546, 1985). describe purification and characterization of endoxylanases isolated from *A. niger*.

WO 91/19782 and EP 463 706 discloses xylanase derived from *Aspergillus niger* origin and the recombinant production thereof. The xylanase is stated to be useful for baking, brewing, in the paper making industry, and in the treatment of agricultural waste, etc.

Torronen, A et al. (Biotechnology 10:1461-1465, 1992) decribe cloning and characterization of two xylanases from Trichoderma reesei and Haas, H et al.(Gene 126:237-242, 1992) describe cloning of a xylanase from *Penicillum chrysogenum*.

SUMMARY OF THE INVENTION

According to the present invention, the inventors have now succeeded in isolating and characterizing a DNA sequence, which encodes an enzyme exhibiting xylanase activity, thereby making it possible to prepare a mono-component xylanase preparation.

Accordingly, in a first aspect the invention relates to a DNA construct comprising a DNA sequence encoding an enzyme exhibiting xylanase activity, which DNA sequence comprises

(a) the xylanase encoding part of the DNA sequence shown in SEQ ID No. 1, or

b) an analogue of the DNA sequence defined in a) which

i) is homologous with the DNA sequence defined in a), or

ii) hybridizes with the same nucleotide probe as the DNA sequence defined in a), or

iii) encodes a polypeptide which is at least 70% homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence defined in a), or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified xylanase encoded by the DNA sequence defined in a).

The full length DNA sequence SEQ ID No. 1 encoding a xylanase has been derived from a strain of the filamentous fungus *Thielavia terrestris* and is present in the *Escherichia coli* strain DSM No. 10363. The xylanase encoding sequence harboured in DSM 10363 is believed to have the same sequence as that identified in SEQ ID NO 1. Accordingly, whenever reference is made to the xylanase encoding part of SEQ ID No. 1 such reference is also intended to include reference to the xylanase encoding DNA sequence present in DSM 10363. Accordingly, the terms "the xylanase encoding part of the DNA sequence SEQ ID No. 1" and "the xylanase encoding DNA sequence present in DSM 10363" may be used interchangeably.

In further aspects the invention provides an expression vector harbouring the DNA construct of the invention, a cell

comprising said DNA construct or said expression vector and a method of producing an enzyme exhibiting xylanase activity, which method comprises culturing said cell under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

In a still further aspect the invention provides an enzyme exhibiting xylanase activity, which enzyme,

(a) is encoded by a DNA construct of the invention; or

(b) is produced by the method of the invention; and/or

(c) is immunologically reactive with an antibody raised against a purified xylanase encoded by the DNA sequence obtainable from *Escherichia coli* DSM no. 10363.

In a still further aspect, the present invention provides an enzyme preparation useful for the degradation or modification of plant material or components, said preparation being enriched in an enzyme exhibiting xylanase activity as described above.

In a still further aspect, the present invention relates to the use of an enzyme or an enzyme preparation of the invention for various industrial applications.

Finally the invention relates to an isolated substantially pure biological culture of the *E. coli* strain DSM No. 10363 harbouring a xylanase encoding DNA sequence (the xylanase encoding part of the SEQ ID No. 1) derived from a strain of the filamentous fungus *Thielavia terrestris* or any mutant of said strain having retained the xylanase encoding capability; and to an isolated substantially pure biological culture of the filamentous fungus *Thielavia terrestris* NRRL No. 8126, from which the DNA sequence presented as SEQ ID No. 1 has been derived.

DETAILED DESCRIPTION OF THE INVENTION

DNA Constructs

The present invention provides a DNA construct comprising a DNA sequence encoding an enzyme exhibiting xylanase

activity, which DNA sequence comprises

(a) the xylanase encoding part of the DNA sequence shown in SEQ ID No. 1, or

(b) an analogue of the DNA sequence defined in a) which

(i) is homologous with the DNA sequence defined in (a), or

(ii) hybridizes with the same nucleotide probe as the DNA sequence defined in (a), or

(iii) encodes a polypeptide which is at least 70% homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence defined in (a), or

(iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified xylanase encoded by the DNA sequence defined in (a).

As used herein the term "xylanase encoding part" used in connection with a DNA sequence means the region of the DNA sequence which corresponds to the region which is translated into a polypeptide sequence. In the DNA sequence shown in SEQ ID NO 1 it is the region between the first "ATG" start codon ("AUG" codon in mRNA) and the following stop codon ("TAA", "TAG" or "TGA"). In others words this is the translated polypeptide.

The translated polypeptide comprises, in addition to the mature sequence exhibiting xylanase activity, an N-terminal signal sequence. The signal sequence generally guides the secretion of the polypeptide. For further information see (Stryer, L., "Biochemistry" W.H., Freeman and Company/New York, ISBN 0-7167-1920-7).

In the present context the term "xylanase encoding part" is intended to cover the translated polypeptide and the mature part thereof.

As defined herein, a DNA sequence analogous to the

xylanase encoding part of the DNA sequence SEQ ID No. 1 is intended to indicate any DNA sequence encoding an enzyme exhibiting xylanase activity, which enzyme has one or more of the properties cited under (i)-(iv) above.

The analogous DNA sequence may be isolated from a strain of the filamentous fungus *Thielavia terrestris* producing the enzyme with xylanase activity, or another or related organism and thus, *e.g.* be an allelic or species variant of the xylanase encoded by the DNA sequence SEQ ID No. 1.

Alternatively, the analogous sequence may be constructed on the basis of the DNA sequence presented as the xylanase encoding part of SEQ ID No. 1, *e.g* be a sub-sequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the xylanase encoded by the DNA sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence.

When carrying out nucleotide substitutions, amino acid changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine). For a general description of nucleotide substitution, see *e.g.* Ford et al., (1991), Protein Expression

and Purification 2, 95-107.

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (cf. e.g. Cunningham and Wells, (1989), Science 244, 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (i.e. xylanase) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (cf. e.g. de Vos et al., (1992), Science 255, 306-312; Smith et al., (1992), J. Mol. Biol. 224, 899-904; Wlodaver et al., (1992), FEBS Lett. 309, 59-64).

The xylanase encoded by the DNA sequence of the DNA construct of the invention may comprise a cellulose binding domain (CBD) existing as an integral part of the encoded enzyme, or a CBD from another origin may be introduced into the xylanase enzyme. Examples of suitable CBD´s are given by Tomme, P. et al. ("Cellulose-Binding Domains: Classification and Properties" in "Enzymatic Degradation of Insoluble Carbohydrates", John N. Saddler and Michael H. Penner (Eds.), ACS Symposium Series, No. 618, 1996.). WO93/21331 discloses a suitable method of introducing a CBD into the xylanase of the invention.

It is presently believed that the sequence shown as amino acid number 266 to 295 in SEQ ID No 2 is a cellulose binding domain (CBD). The sequence is following: "(266) WGQCGGQGWTGPTCCSQGTCKAQNQWYSQC(295)" and it is presently believed to be a CBD based on the in the art known CBD consensus sequence "WGQCGGXGXXGXXXCXXGXTCXXXNXXYXQC".

The homology referred to in i) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity preferably of at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95% with the xylanase encoding part of the DNA sequence shown in SEQ ID No. 1.

The hybridization referred to in (ii) above is intended to indicate that the analogous DNA sequence hybridizes to the same probe as the DNA sequence encoding the xylanase enzyme under certain specified conditions which are described in detail in the Materials and Methods section hereinafter. The probe to be used may conveniently be constructed on the basis of the xylanase encoding part of the DNA sequence SEQ ID No. 1, or a sub-sequence thereof encoding at least 6-7 amino acids of the enzyme. In the latter case the probe is prepared from an amino acid subsequence corresponding to a high number of low degenerated codons.

The homology referred to in iii) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the polypeptide encoded by an analogous DNA sequence exhibits a degree of identity preferably of at least 70%, more preferably at least 80%, especially at least 90% with the enzyme encoded by a DNA construct comprising

the xylanase encoding part of the DNA sequence shown in SEQ ID No. 1.

In connection with property iv) the immunological reactivity may be determined by the method described in the Materials and Methods section below.

The DNA sequence encoding a xylanase of the invention can be isolated from the *Escherichia coli* strain *Escherichia coli* DSM No. 10363 using standard methods *e.g.* as described by Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

The DNA sequence encoding an enzyme exhibiting xylanase activity of the invention can also be isolated by any general method involving

- cloning, in suitable vectors, a cDNA library from any organism expected to produce the xylanase of interest,
- transforming suitable yeast host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the cDNA library,
- screening for positive clones by determining any xylanase activity of the enzyme produced by such clones, and
- isolating the enzyme encoding DNA from such clones.

A general isolation method has been disclosed in WO 93/11249 or WO 94/14953, the contents of which are hereby incorporated by reference. A more detailed description of the screening method is given in Example 1 below.

Microbial Sources

In a preferred embodiment, the DNA sequence encoding the xylanase is derived from a strain of *Thielavia,* especially a strain of *Thielavia terrestris*.

It is at present contemplated that a DNA sequence encoding an enzyme homologous to the enzyme of the invention,

i.e. an analogous DNA sequence, may be obtained from other microorganisms. For instance, the DNA sequence may be derived by similarly screening a cDNA library of another microorganism, in particular a fungus, such as a strain of an *Aspergillus sp.*, in particular a strain of *A. aculeatus* or *A. niger*, a strain of *Trichoderma sp.*, in particular a strain of *T. reesei*, *T. viride*, *T. longibrachiatum*, *T. harzianum* or *T. koningii* or a strain of a *Fusarium sp.*, in particular a strain of *F. oxysporum*, or a strain of a *Humicola sp.*, or a strain of a *Neocallimastix sp.*, a *Piromyces sp.*, a *Penicillium sp.*, an *Aureobasidium sp.*, *a Thermoascus sp.*, *a Paecilomyces sp.*, *a Talaromyces sp.*, *a Magnaporthe sp.*, *a Schizophyllum sp.*, *a Filibasidium sp.*, *or a Cryptococcus sp.*

The expression plasmid pYES 2.0 comprising the full length DNA sequence encoding the xylanase of the invention has been transformed into a strain of the *E. coli* which was deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutshe Sammlung von Mikroorganismen und Zellkulturen GmbH.

Deposit date	: 06.12.95
Depositor's ref.	: NN049150
DSM designation	: *Escherichia coli* DSM No. 10363

The DNA sequence encoding the enzyme exhibiting xylanase activity can for instance be isolated from the above mentioned deposited strains by standard methods.

Alternatively, the DNA encoding a xylanase of the invention may, in accordance with well-known procedures, conveniently be isolated from a suitable source, such as any of the above mentioned organisms, by use of synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of the xylanase encoding part of the nucleotide sequences presented as SEQ ID No. 1 or any

suitable subsequence thereof.

Expression vectors

In another aspect, the invention provides a recombinant expression vector comprising the DNA construct of the invention.

The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the expression vector, the DNA sequence encoding the xylanase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the xylanase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (*McKnight et al.*, The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral a-amylase, *Aspergillus niger* acid stable a-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae*

triose phosphate isomerase or *Aspergillus nidulans* acetamidase.

Host cells

In yet another aspect the invention provides a host cell comprising the DNA construct of the invention and/or the recombinant expression vector of the invention.

Preferably, the host cell of the invention is a eukaryotic cell, in particular a fungal cell such as a yeast or filamentous fungal cell. In particular, the cell may belong to a species of *Trichoderma*, preferably *Trichoderma harzianum* or *Trichoderma reesei*, or a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference. The host cell may also be a yeast cell, e.g. a strain of *Saccharomyces*, in particular *Saccharomyces* cerevisae, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp., *Pichia* sp., *Yarrowia* sp., such as *Yarrowia lipolytica*, or *Kluyveromyces* sp., such as *Kluyveromyces lactis*.

Method of producing xylanase

In a still further aspect, the present invention provides a method of producing an enzyme according to the invention, wherein a suitable host cell, which has been transformed with a DNA sequence encoding the enzyme, is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed xylanase may conveniently be secreted into the culture medium and may be recovered therefrom

by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Enzyme compositions

In a still further aspect, the present invention relates to an enzyme preparation useful for the degradation of plant cell wall components, said preparation being enriched in an enzyme exhibiting xylanase activity as described above. In this manner a boosting of the cell wall degrading ability of the enzyme preparation can be obtained.

The enzyme composition having been enriched with an enzyme of the invention may e.g. be an enzyme preparation comprising multiple enzymatic activities, in particular an enzyme preparation comprising multiple plant cell wall degrading enzymes such as Biofeed+®, Energex®, Viscozym®, Pectinex®, Pectinex Ultra SP®, (all available from Novo Nordisk A/S). In the present context, the term "enriched" is intended to indicate that the xylanase activity of the enzyme preparation has been increased, e.g. with an enrichment factor of 1.1, conveniently due to addition of an enzyme of the invention prepared by the method described above.

Alternatively, the enzyme preparation enriched in an enzyme exhibiting xylanase activity may be one which comprises an enzyme of the invention as the major enzymatic component, e.g. a mono-component enzyme preparation.

The enzyme preparation may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry preparation. For instance, the enzyme preparation may be in the form of a granulate or a microgranulate. The enzyme to be included in the preparation may be stabilized in accordance with methods known in the art.

The enzyme preparation of the invention may, in addition

to a xylanase of the invention, contain one or more other enzymes, for instance those with xylanolytic, or pectinolytic activities such as a-arabinosidase, a-glucoronisidase, b-xylosidase, xylan acetyl esterase, arabinanase, rhamnogalacturonase, pectin acetylesterase, galactanase, pectin lyase, pectate lyase, glucanase, pectin methylesterase. The additional enzyme(s) may be producible by means of a microorganism belonging to the genus *Aspergillus*, preferably *Aspergillus niger*, *Aspergillus aculeatus*, *Aspergillus awamori* or *Aspergillus oryzae*, or *Trichoderma*, or *Humicola insolens*. Examples are given below of preferred uses of the enzyme preparation of the invention. The dosage of the enzyme preparation of the invention and other conditions under which the preparation is used may be determined on the basis of methods known in the art. In general terms, the enzyme is to be used in an efficient amount for providing the desired effect.

The enzyme preparation according to the invention may be useful for at least one of the following purposes.

Degradation or modification of plant material

The enzyme preparation according to the invention is preferably used as an agent for degradation or modification of plant cell walls or any xylan-containing material originating from plant cells walls due to the high plant cell wall degrading activity of the xylanase of the invention.

The xylanase of the invention hydrolyse b-1,4 linkages in xylans. Xylans are polysaccharides having a backbone composed of b-1,4 linked xylose. The backbone may have different sidebranches, like arabinose, acetyl, glucuronic acid and 4-methylglucuronic acid sidebranches. The composition and number of sidebranches vary according to the source of the xylan. Arabinose sidebranches dominate in xylans from cereal endosperm, whereas xylans from hard wood contain relatively more acetyl and glucuronic acid substituents (Michael P. Coughlan and Geoffrey P. Hazlewood. Biotechnol.Appl. Biochem. 17 : 259-289 (1993). Xylan originating from red algae contains a mixture of b-1,4 and

b-1,3 linked xylose in the backbone, this type of xylan is degradable by xylanases to varying extent due to the 1,4-links in the backbone.

The degradation of xylan by xylanases is facilitated by full or partial removal of the sidebranches. Acetyl groups can be removed by alkali, or by xylan acetyl-esterases, arabinose sidegroups can be removed by a mild acid treatment or by alpha-arabinosidases and the glucuronic acid sidebranches can be removed by alpha-glucuronisidases. The oligomers with are released by the xylanase or by a combination of xylanases and sidebranch-hydrolysing enzymes as mentioned above can be further degraded to free xylose by beta-xylosidases.

The xylanase of the present invention can be used without other xylanolytic enzymes or with limited activity of other xylanolytic enzymes to degrade xylans for production of oligosaccharides. The oligosaccharides may be used as bulking agents, like arabinoxylan oligosaccharides released from cereal cell wall material, or of more or less purified arabinoxylans from cereals.

The xylanase of the present invention can be used in combination with other xylanolytic enzymes to degrade xylans to xylose and other monosaccharides. The released xylose may be converted to other compounds like furanone flavours.

The xylanase of the present invention may be used alone or together with other enzymes like a glucanase to improve the extraction of oil from oil-rich plant material, like corn-oil from corn-embryos.

The xylanase of the present invention may be used for separation of components of plant cell materials, in particular of cereal components such as wheat components. Of particular interest is the separation of wheat into gluten and starch, i.e. components of considerable commercial interest. The separation process may be performed by use of methods known in the art, conveniently a so-called batter process (or wet milling process) performed as a hydroclone or a decanter process. In the batter process, the starting material is a dilute pumpable dispersion

of the plant material such as wheat to be subjected to separation. In a wheat separation process the dispersion is made normally from wheat flour and water.

The xylanase of the invention may also be used in the preparation of fruit or vegetable juice in order to increase yield, and in the enzymatic hydrolysis of various plant cell wall-derived materials or waste materials, e.g. from paper production, or agricultural residues such as wheat-straw, corn cobs, whole corn plants, nut shells, grass, vegetable hulls, bean hulls, spent grains, sugar beet pulp, olive pulp, and the like.

The plant material may be degraded in order to improve different kinds of processing, facilitate purification or extraction of other component than the xylans like purification of beta-glucan or beta-glucan oligomers from cereals, improve the feed value, decrease the water binding capacity, improve the degradability in waste water plants, improve the conversion of e.g. grass and corn to ensilage, etc.

Finally, the xylanase of the invention may be used in modifying the viscosity of plant cell wall derived material. For instance, the xylanase may be used to reduce the viscosity of feed containing xylan, to promote processing of viscous xylan containing material as in wheat separation, and to reduce viscosity in the brewing process.

Preparation of dough or baked product

The xylanase of the present invention may be used in baking so as to improve the development, elasticity and/or stability of dough and/or the volume, crumb structure and/or anti-staling properties of the baked product. The xylanase may be used for the preparation of dough or baked products prepared from any type of flour or meal (e.g. based on rye, barley, oat, or maize), particularly in the preparation of dough or baked products made from wheat or comprising substantial amounts of wheat. The baked products produced with an xylanase of the invention includes bread, rolls, baquettes and the like. For

baking purposes the xylanase of the invention may be used as the only or major enzymatic activity, or may be used in combination with other enzymes such as a lipase, an amylase, an oxidase (e.g. glucose oxidase, peroxidase), a laccase and/or a protease.

Animal Feed Additives

The xylanase of the present invention may be used for modification of animal feed and may exert their effect either *in vitro* (by modifying components of the feed) or *in vivo*. The xylanase is particularly suited for addition to animal feed compositions containing high amounts of arabinoxylans and glucuronoxylans, e.g. feed containing cereals such as barley, wheat, rye or oats or maize. When added to feed the xylanase significantly improves the *in vivo* break-down of plant cell wall material partly due to a reduction of the intestinal viscosity (Bedford et al., 1993), whereby a better utilization of the plant nutrients by the animal is achieved. Thereby, the growth rate and/or feed conversion ratio (i.e. the weight of ingested feed relative to weight gain) of the animal is improved.

Paper and pulp industry

The xylanase of the present invention may be used in the paper and pulp industry, inter alia in the bleaching process to enhance the brightness of bleached pulps whereby the amount of chlorine used in the bleaching stages may be reduced, and to increase the freeness of pulps in the recycled paper process (Eriksson, K.E.L., Wood Science and Technology 24 (1990): 79-101; Paice, et al., Biotechnol. and Bioeng. 32 (1988): 235-239 and Pommier et al., Tappi Journal (1989): 187-191). Furthermore, the xylanase may be used for treatment of lignocellulosic pulp so as to improve the bleachability thereof. Thereby the amount of chlorine needed to obtain a satisfactory bleaching of the pulp may be reduced. The treament of lignocellulosic pulp may, e.g., be performed as described in WO 93/08275, WO 91/02839 and WO 92/03608.

Beer brewing

The xylanase of the present invention may be used in beer brewing, in particular to improve the filterability of wort e.g. containing barley and/or sorghum malt. The xylanase may be used in the same manner as pentosanases conventionallly used for brewing, e.g. as described by Viëtor et al., 1993 and EP 227 159. Furthermore, the xylanase may be used for treatment of brewers spent grain, i.e. residuals from beer wort production containing barley or malted barley or other cereals, so as to improve the utilization of the residuals for, e.g., animal feed.

The invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

MATERIALS AND METHODS

Deposited organisms

Thielavia terrestris NRRL 8126 comprises the xylanase encoding DNA sequence of the invention.

Escherichia coli DSM 10363 containing the plasmid comprising the full length DNA sequence, coding for the xylanase of the invention, in the shuttle vector pYES 2.0.

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (MATa; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; cir+).

Plasmids

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (Invitrogen)

pA2X154 (See example 1)

General molecular biology methods

Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of

molecular biology (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990).

Enzymes for DNA manipulations were used according to the specifications of the suppliers.

Enzymes for DNA manipulations

Unless otherwise mentioned all enzymes for DNA manipulations, such as *e.g.* restiction endonucleases, ligases etc., are obtained from New England Biolabs, Inc.

Expression cloning in yeast

Expression cloning in yeast was done as comprehensively described by H. Dalboege et al. (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953), which are hereby incorporated as reference.

The individual steps in the expression cloning technique according to the references above are further described below.

Isolation of the DNA sequence shown in SEQ ID No. 1

The xylanase encoding part of the DNA sequence shown in SEQ ID No. 1 coding for the xylanase of the invention can be obtained from the deposited organism *Escherichia coli* DSM 10363 by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

Extraction of total RNA was performed with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion, and isolation of poly(A)$^+$RNA was carried out by oligo(dT)-cellulose affinity chromatography using the procedures described in WO 94/14953.

cDNA synthesis: Double-stranded cDNA was synthesized

from 5 mg poly(A)$^+$ RNA by the RNase H method (Gubler and Hoffman (1983) Gene **25**:263-269, Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY) using the hair-pin modification developed by F. S. Hagen (pers. comm.). The poly(A)$^+$ RNA (5 mg in 5 ml of DEPC-treated water) was heated at 70°C for 8 min. in a pre-siliconized, RNase-free Eppendorph tube, quenched on ice and combined in a final volume of 50 ml with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, Bethesda Research Laboratories) containing 1 mM of dATP, dGTP and dTTP and 0.5 mM 5-methyl-dCTP (Pharmacia), 40 units human placental ribonuclease inhibitor (RNasin, Promega), 1.45 mg of oligo(dT)$_{18}$-Not I primer (Pharmacia) and 1000 units SuperScript II RNase H reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA was synthesized by incubating the reaction mixture at 45°C for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture was gelfiltrated through a MicroSpin S-400 HR (Pharmacia) spin column according to the manufacturer's instructions.

After the gelfiltration, the hybrids were diluted in 250 ml second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.16 mM bNAD+) containing 200 mM of each dNTP, 60 units *E. coli* DNA polymerase I (Pharmacia), 5.25 units RNase H (Promega) and 15 units *E. coli* DNA ligase (Boehringer Mannheim). Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 2 hours and additional 15 min. at 25°C. The reaction was stopped by addition of EDTA to a final concentration of 20 mM followed by phenol and chloroform extractions.

Mung bean nuclease treatment

The double-stranded cDNA was precipitated at -20°C for 12 hours by addition of 2 vols 96% EtOH, 0.2 vol 10 M NH_4Ac, recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 ml Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM $ZnSO_4$, 0.35 mM DTT, 2% glycerol)

containing 25 units Mung bean nuclease (Pharmacia). The single-stranded hair-pin DNA was clipped by incubating the reaction at 30°C for 30 min., followed by addition of 70 ml 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction and precipitation with 2 vols of 96% EtOH and 0.1 vol 3 M NaAc, pH 5.2 on ice for 30 min.

Blunt-ending with T4 DNA polymerase

The double-stranded cDNAs were recovered by centrifugation and blunt-ended in 30 ml T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM of each dNTP and 5 units T4 DNA polymerase (New England Biolabs) by incubating the reaction mixture at 16°C for 1 hour. The reaction was stopped by addition of EDTA to a final concentration of 20 mM, followed by phenol and chloroform extractions, and precipitation for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Adaptor ligation, Not I digestion and size selection

After the fill-in reaction the cDNAs were recovered by centrifugation, washed in 70% EtOH and dried. The cDNA pellet was resuspended in 25 ml ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) containing 2.5 mg non-palindromic BstXI adaptors (Invitrogen) and 30 units T4 ligase (Promega) and incubated at 16°C for 12 hours. The reaction was stopped by heating at 65°C for 20 min. and then cooling on ice for 5 min. The adapted cDNA was digested with Not I restriction enzyme by addition of 20 ml water, 5 ml 10x Not I restriction enzyme buffer (New England Biolabs) and 50 units Not I (New England Biolabs), followed by incubation for 2.5 hours at 37°C. The reaction was stopped by heating at 65°C for 10 min. The cDNAs were size-fractionated by gel electrophoresis on a 0.8% SeaPlaque GTG low melting temperature agarose gel (FMC) in 1x TBE to separate unligated adaptors and small cDNAs. The cDNA was size-selected with a cut-off at 0.7 kb and rescued from the gel by use of b-Agarase (New England Biolabs) according to the manufacturer's instructions and precipitated for 12 hours at -

20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Construction of libraries

The directional, size-selected cDNA was recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 ml 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The cDNAs were desalted by gelfiltration through a MicroSpin S-300 HR (Pharmacia) spin column according to the manufacturer's instructions. Three test ligations were carried out in 10 ml ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) containing 5 ml double-stranded cDNA (reaction tubes #1 and #2), 15 units T4 ligase (Promega) and 30 ng (tube #1), 40 ng (tube #2) and 40 ng (tube #3, the vector background control) of BstXI-NotI cleaved pYES 2.0 vector. The ligation reactions were performed by incubation at 16°C for 12 hours, heating at 70°C for 20 min. and addition of 10 ml water to each tube. 1 ml of each ligation mixture was electroporated into 40 ml electrocompetent *E. coli* DH10B cells (Bethesda research Laboratories) as described (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). Using the optimal conditions a library was established in *E. coli* consisting of pools. Each pool was made by spreading transformed *E. coli* on LB+ampicillin agar plates giving 15.000-30.000 colonies/plate after incubation at 37°C for 24 hours. 20 ml LB+ampicillin was added to the plate and the cells were suspended herein. The cell suspension was shaked in a 50 ml tube for 1 hour at 37°C. Plasmid DNA was isolated from the cells according to the manufacturer's instructions using QIAGEN plasmid kit and stored at -20°C.

1 ml aliquots of purified plasmid DNA (100 ng/ml) from individual pools were transformed into *S. cerevisiae* W3124 by electroporation (Becker and Guarante (1991) Methods Enzymol. **194**:182-187) and the transformants were plated on SC agar containing 2% glucose and incubated at 30°C.

Identification of positive clones

The tranformants was plated on SC agar containing 0.1% AZCL xylan (Megazyme, Australia) and 2% Galactose and incubated for 3-5 days at 30 C.

Xylanase positive colonies are identified as colonies surrounded by a blue halo.

Characterization of positive clones

The positive clones were obtained as single colonies, the cDNA inserts were amplified directly from the yeast colony using biotinylated polylinker primers, purified by magnetic beads (Dynabead M-280, Dynal) system and characterized individually by sequencing the 5'-end of each cDNA clone using the chain-termination method (Sanger et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467) and the Sequenase system (United States Biochemical).

Isolation of a cDNA gene for expression in *Aspergillus*

A xylanase-producing yeast colony was inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

DNA was isolated according to WO 94/14953 and dissolved in 50 ml water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis. The cDNA insert was excised using appropriate restriction enzymes and ligated into an Aspergillus expression vector.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

Protoplasts may be prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12, which is hereby incorporated by reference.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$). Protoplasts are mixed with p3SR2

(an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the transformants were inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation at 30°C, the supernatant was removed. The xylanolytic activity was identified by applying 10 µl supernatant to 4 mm diameter holes punched out in agar plates containing 0.2% AZCL® birch xylan (Megazyme®, Australia). Xylanolytic activity is then identified as a blue halo.

Hybridization conditions

Suitable hybridization conditions for determining hybridization between an oligonucleotide probe and an "analogous" DNA sequence of the invention may be defined as described below. A suitable oligonucleotide probe to be used in the hybridization may be prepared on the basis of the xylanase encoding part of the DNA sequence shown in SEQ ID No. 1, or any sub-sequence thereof. An example of a suitable probe, is the DNA sequence corresponding to the xylanase encoding part of SEQ ID No. 1.

Hybridization

The hybridization referred to above is intended to comprise an analogous DNA sequence which hybridizes to the nucleotide probe corresponding to the xylanase encoding part of the DNA sequence shown in SEQ ID NO 1, i.e. nucleotides 1-894, under at least under at least low stringency conditions and preferably at medium or high stringency conditions as described in detail below.

Suitable experimental conditions for determining hybridization at low, medium, or high stringency between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al. 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at least 55°C (low stringency), more preferably at least 60°C (medium stringency), still more preferably at least 65°C (medium/high stringency), even more preferably at least 70°C (high stringency), even more preferably at least 75°C (very high stringency).

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

Immunological cross-reactivity

Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified xylanase. More specifically, antiserum against the xylanase of the invention may be raised by immunizing rabbits (or other rodents)

according to the procedure described by N. Axelsen et al. in: A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically p. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2$ SO_4), followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

Media

YPD: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

10 x Basal salt: 75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H_2O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

SC-agar: SC-URA, 20 g/l agar added.

SC-variant agar: 20 g agar, 20 ml 10 x Basal salt, H_2O ad 900 ml, autoclaved

AZCL xylan (Megazyme, Australia)

PEG 4000 (polyethylene glycol, molecular weight = 4,000) (BDH, England)

EXAMPLES

EXAMPLE 1

Cloning and expression of a xylanase from *Thielavia terrestris* NRRL No. 8126

mRNA was isolated from *Thielavia terrestris*, NRRL No. 8126, grown in cellulose-containing fermentation medium with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *Thielavia terrestris*, NRRL No. 8126, consisting of approx. $9x10^5$ individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Xylanase-positive colonies were identified and isolated on SC-agar plates with the AZCL xylan assay. cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above. The DNA sequence of the cDNA encoding the xylanase is shown in SEQ ID No. 1.

The cDNA is obtainable from the plasmid in DSM 10363.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the xylanase in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the xylanase gene was purified. The gene was subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmid pA2X154.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

Test of *A. oryzae* transformants

Each of the transformants were tested for enzyme

activity as described above. Some of the transformants had xylanase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the xylanase in *Aspergillus oryzae*.

EXAMPLE 2

A homology search with the xylanase of the invention against nucleotide and protein databases was performed. The homology search showed that the most related xylanases were xylanase II from Trichoderma reesei and xylanase A from Aspergillus nidulans. The xylanase from Trichoderma reesei belongs to family 11 of glycosyl hydrolases which indicate that the xylanase of the invention also belongs to family 11 of glycosyl hydrolases (Henrissat, B Biochem. J. 280:309-316, 1991).

According to the method described in the "DETAILED DESCRIPTION OF THE INVENTION" the DNA homology of the xylanase of the invention against most prior art xylanases was determined using the computer program GAP. The xylanase of the invention has only 61% DNA homology to the xylanase II from Trichoderma reesei (Torronen, A. et al., Biotechnology (N.Y.) 10 (11), 1461-1465(1992)) and the xylanase of the invention has only 56% DNA homology to xylanase A from Aspergillus nidulans (ACCESSION No. Z49892, Genebank). This show that the xylanase of the invention indeed is distant from any known xylanases.

SEQUENCE LISTING

SEQ ID No. 1 shows the DNA sequence of the full-length DNA sequence comprised in the DNA construct transformed into the deposited *Escherichia coli* DSM 10363.

2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 894 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Thielavia terrestris
(B) STRAIN: NRRL 8126

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..891

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATG GTT GGC TTC TCG AAC ATC GTC CTT GGC CTT TCG GCC GCC GCG GCA      48
Met Val Gly Phe Ser Asn Ile Val Leu Gly Leu Ser Ala Ala Ala Ala
 1               5                  10                  15

ACC CTC GCG GCT CCC ACC GCC GAG CGC GGC GCG GCT AAC TTC GTC CTG      96
Thr Leu Ala Ala Pro Thr Ala Glu Arg Gly Ala Ala Asn Phe Val Leu
            20                  25                  30

CAC CCT GAT CAT CCT CTG GCC CGC CGC ATC GGC AAC CTG ACG GCG CGC     144
His Pro Asp His Pro Leu Ala Arg Arg Ile Gly Asn Leu Thr Ala Arg
        35                  40                  45
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TCC AAC CCG AGC TAC ACG CAG AAC TAC CAG ACT GGC GGC ACC GTC AAC      192
Ser Asn Pro Ser Tyr Thr Gln Asn Tyr Gln Thr Gly Gly Thr Val Asn
     50                  55                  60

TTC ACC CCC ACG GGC ACC GGC TTC ACG CTG AAC TAC AAT GTC CAG CAG      240
Phe Thr Pro Thr Gly Thr Gly Phe Thr Leu Asn Tyr Asn Val Gln Gln
 65                  70                  75                  80

GAC TTT GTT GTC GGC GTC GGC TGG AAC CCC GGC AGC AAC CAG CCC ATC      288
Asp Phe Val Val Gly Val Gly Trp Asn Pro Gly Ser Asn Gln Pro Ile
                 85                  90                  95

ACC CAC TCG GGC ACC TTC ACC GTC AAC AGC GGT CTG GGC AGT CTC AGC      336
Thr His Ser Gly Thr Phe Thr Val Asn Ser Gly Leu Gly Ser Leu Ser
            100                 105                 110

GTG TAC GGC TGG AGC ACG AAC CCG CTG GTG GAG TAC TAC ATC ATG GAG      384
Val Tyr Gly Trp Ser Thr Asn Pro Leu Val Glu Tyr Tyr Ile Met Glu
        115                 120                 125

GTG AAC GAC GGC ATC ACG GTG GGC GGG CAG CAG ATG GGC ACG GTG GAG      432
Val Asn Asp Gly Ile Thr Val Gly Gly Gln Gln Met Gly Thr Val Glu
    130                 135                 140

AGC GAC GGC GGC ACT TAC ACC ATC TGG AAG CAC CAG CAG GTG AAC CAG      480
Ser Asp Gly Gly Thr Tyr Thr Ile Trp Lys His Gln Gln Val Asn Gln
145                 150                 155                 160

CCG GCC ATC GCC GGG TCG GGC CTG TAC ACG TTC TGG CAG TAC ATC TCG      528
Pro Ala Ile Ala Gly Ser Gly Leu Tyr Thr Phe Trp Gln Tyr Ile Ser
                165                 170                 175

ATC CGC GAC TCG CCG CGC ACG AGC GGC ACC GTC ACG GTG CAG AAC CAC      576
Ile Arg Asp Ser Pro Arg Thr Ser Gly Thr Val Thr Val Gln Asn His
            180                 185                 190

TTC GAC GCC TGG GCG AAG CTC GGC ATG AAC CTC GGC ACC ATG AAC CTG      624
Phe Asp Ala Trp Ala Lys Leu Gly Met Asn Leu Gly Thr Met Asn Leu
        195                 200                 205

CAG GTC GTC GCC GTC GAG AGC TGG AGC GGC AGC GGC TCC GCC CAG CAG      672
Gln Val Val Ala Val Glu Ser Trp Ser Gly Ser Gly Ser Ala Gln Gln
    210                 215                 220
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ACC GTG TAC AAC GGC GGC TCG GGC AGC ACT GGC GGC AGC GGC GGC GGC      720
Thr Val Tyr Asn Gly Gly Ser Gly Ser Thr Gly Gly Ser Gly Gly Gly
225                 230                 235                 240

AAT GGT GGC AGC AGC GGC GGC AAT GGT GGC AGC AGC GGC GGC AGC GGC      768
Asn Gly Gly Ser Ser Gly Gly Asn Gly Gly Ser Ser Gly Gly Ser Gly
                245                 250                 255

GGC AGC ACC GGC ACC TGC TCC GCG CTC TGG GGC CAG TGC GGC GGC CAG      816
Gly Ser Thr Gly Thr Cys Ser Ala Leu Trp Gly Gln Cys Gly Gly Gln
            260                 265                 270

GGC TGG ACC GGC CCG ACC TGC TGC TCC CAG GGC ACC TGC AAG GCC CAG      864
Gly Trp Thr Gly Pro Thr Cys Cys Ser Gln Gly Thr Cys Lys Ala Gln
        275                 280                 285

AAC CAG TGG TAC TCG CAG TGC CTG CAG TAA                              894
Asn Gln Trp Tyr Ser Gln Cys Leu Gln
    290                 295
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 297 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Val Gly Phe Ser Asn Ile Val Leu Gly Leu Ser Ala Ala Ala Ala
 1               5                  10                  15

Thr Leu Ala Ala Pro Thr Ala Glu Arg Gly Ala Ala Asn Phe Val Leu
            20                  25                  30

His Pro Asp His Pro Leu Ala Arg Arg Ile Gly Asn Leu Thr Ala Arg
        35                  40                  45

Ser Asn Pro Ser Tyr Thr Gln Asn Tyr Gln Thr Gly Gly Thr Val Asn
    50                  55                  60

Phe Thr Pro Thr Gly Thr Gly Phe Thr Leu Asn Tyr Asn Val Gln Gln
65                  70                  75                  80

Asp Phe Val Val Gly Val Gly Trp Asn Pro Gly Ser Asn Gln Pro Ile
                85                  90                  95

Thr His Ser Gly Thr Phe Thr Val Asn Ser Gly Leu Gly Ser Leu Ser
            100                 105                 110

Val Tyr Gly Trp Ser Thr Asn Pro Leu Val Glu Tyr Tyr Ile Met Glu
        115                 120                 125

Val Asn Asp Gly Ile Thr Val Gly Gly Gln Gln Met Gly Thr Val Glu
    130                 135                 140

Ser Asp Gly Gly Thr Tyr Thr Ile Trp Lys His Gln Gln Val Asn Gln
145                 150                 155                 160

Pro Ala Ile Ala Gly Ser Gly Leu Tyr Thr Phe Trp Gln Tyr Ile Ser
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                165                 170                 175

Ile Arg Asp Ser Pro Arg Thr Ser Gly Thr Val Thr Val Gln Asn His
            180                 185                 190

Phe Asp Ala Trp Ala Lys Leu Gly Met Asn Leu Gly Thr Met Asn Leu
        195                 200                 205

Gln Val Val Ala Val Glu Ser Trp Ser Gly Ser Gly Ser Ala Gln Gln
    210                 215                 220

Thr Val Tyr Asn Gly Gly Ser Gly Ser Thr Gly Gly Ser Gly Gly Gly
225                 230                 235                 240

Asn Gly Gly Ser Ser Gly Gly Asn Gly Gly Ser Ser Gly Gly Ser Gly
                245                 250                 255

Gly Ser Thr Gly Thr Cys Ser Ala Leu Trp Gly Gln Cys Gly Gly Gln
            260                 265                 270

Gly Trp Thr Gly Pro Thr Cys Cys Ser Gln Gly Thr Cys Lys Ala Gln
        275                 280                 285

Asn Gln Trp Tyr Ser Gln Cys Leu Gln
    290                 295
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 10, line 22-24.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany

Date of deposit	Accession Number
6 December 1995	DSM 10363

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

During the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. e.g. Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71) in those designated states providing for such "expert solution".

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Form PCT/RO/134 (July 1992)

CLAIMS

1. A DNA construct comprising a DNA sequence encoding an enzyme exhibiting xylanase activity, which DNA sequence comprises

(a) the xylanase encoding part of the DNA sequence shown in SEQ ID No. 1, or

(b) an analogue of the DNA sequence defined in a) which

(i) is homologous with the DNA sequence defined in (a), or

(ii) hybridizes with the same nucleotide probe as the DNA sequence defined in (a), or

(iii) encodes a polypeptide which is at least 70% homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence defined in (a), or

(iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified xylanase encoded by the DNA sequence defined in (a).

2. The DNA construct according to claim 1, in which the DNA sequence encoding an enzyme exhibiting xylanase activity is obtainable from a microorganism, preferably a filamentous fungus, a yeast, or a bacteria.

3. The DNA construct according to claim 2, in which the DNA sequence is isolated from or produced on the basis of a DNA library of the strain of *Thielavia*, in particular a strain of *Thielavia terrestris*, especially *Thielavia terrestris*, NRRL 8126.

4. The DNA construct according to claim 2, in which the DNA sequence is obtainable from a strain of *Aspergillus*, *Trichoderma*, *Fusarium*, *Humicola*, *Neocallimastix*, *Piromyces*, *Penicillium*, *Aureobasidium*, *Thermoascus*, *Paecilomyces*,

Talaromyces, Magnaporthe, Schizophyllum, Filibasidium, or a *Cryptococcus*.

5. The DNA construct according to claim 1, in which the DNA sequence is isolated from *Saccharomyces cerevisiae* DSM No. 9978.

6. A recombinant expression vector comprising a DNA construct according to any of claims 1-5.

7. A cell comprising a DNA construct according to any of claims 1-6 or a recombinant expression vector according to claim 6.

8. The cell according to claim 7, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

9. The cell according to claim 8, which is a strain of *Fusarium* or *Aspergillus* or *Trichoderma*, in particular a strain of *Fusarium graminearum, Fusarium cerealis, Aspergillus niger, Aspergillus Oryzae, Trichoderma harzianum* or *Trichoderma reesei*.

10. The cell according to claim 8, which is a strain of *Thielavia* sp., in particular *Thielavia terrestris*.

11. The cell according to claim 10, being the strain *Thielavia terrestris* NRRL No. 8126.

12. A cell according to claim 8, which is a strain of *Saccharomyces*, in particular a strain of *Saccharomyces cerevisiae*.

13. A method of producing an enzyme exhibiting xylanase activity, the method comprising culturing a cell according to any of claims 7-12 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

14. An enzyme exhibiting xylanase activity, which enzyme

(a) is encoded by a DNA construct according to any of claims 1-6, or

(b) produced by the method according to claim 13, and/or

(c) is immunologically reactive with an antibody raised against a purified xylanase encoded by the xylanase encoding part of the DNA sequence shown in SEQ ID No. 1.

15. A composition comprising the enzyme according to claim 14.

16. An enzyme composition which is enriched in an enzyme exhibiting xylanase activity according to claim 14.

17. A composition according to claim 16, which additionally comprises a pectin lyase, pectate lyase, glucanase, xylosidase, arabinosidase, xylan acetyl esterase, or pectin methylesterase.

18. Use of an enzyme according to claim 14 or an enzyme composition according to any of claims 15 to 17 in the production of dough or baked products.

19. Use of a enzyme according to claim 14 or an enzyme preparation according to any of claims 15 to 17 in the preparation of feed or food.

20. Use of an enzyme according to claim 14 or an enzyme preparation according any of claims 15 to 17 in the preparation of pulp or paper.

21. Use of an enzyme according to any of claim 14 or an enzyme preparation according to any of claim 15 to 17 for the

separation of cereal components.

22. The use according to claim 21, in which the cereal is wheat.

23. The use according to claim 21 or 22, in which the cereal component is wheat which is to be separated into gluten and starch.

24. Use of en enzyme according to claim 14 or an enzyme preparation according to any of claims 15 to 17 for reducing the viscosity of a plant cell wall derived material.

25. Use of an enzyme according to any of claim 14 or an enzyme preparation according to any of claims 15 to 17 in the production of beer or modification of by-products from a brewing process.

26. Use of an enzyme according to any of claim 14 or an enzyme preparation according to any of claims 15 to 17 in the production of wine or juice.

27. An isolated substantially pure biological culture of the deposited strain *Escherichia coli* DSM No. 10363.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00033

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/24
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, EMBL/GENBANK/DDBJ, SWISSPROT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL, Databas Genbank/DDBJ, accession no. X69574, Toerroenen A. et al: "The two major xylanases from trichoderma reesei: characterization of both enzymes and genes"; & Biotechnology (N.Y.) 10:1461-146 (1992), 1993-08-10 ---	1-27
X	EMBL, Databas Genbank/DDBJ, accession no. Q54776, ALKO OY AB: "Isolated nucleic acid mol. used in enzymes for paper, pulp and feed industry - comprising sequence encoding aminoacid sequence of T. reesei pI 5.5 xylanase"; & WO,A,9324621, 1994-06-10 ---	1-27

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 April 1997	08.05.1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00033

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 07162880, Medline accession no. 92321766, Gilbert M et al: "Purification and characterization of a xylanase from the thermophilic ascomycete Thelavia terrestris 255B"; & Appl Biochem Biotechnol (UNITED STATES) Spring 1992,34-35, p247-59	1-27
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X	Dialog Information Services, File 5, BIOSIS PREVIEWS(R), Dialog accession no. 5912948, Biosis no. 84045513, Grajek W: "Production of D xylanase by thermophilic fungi using different methods of culture; & Biotechnol Lett 9 (5)	1-27
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X	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 07813761, Medline accession no. 94114174, Gilbert M et al: "A comparison of two xylanases from the thermophilic fungi Thielavia terrestris and Thermoascus crustaceus"; & Appl Microbiol Biotechnol (GERMANY) Dec 1993, 40 (4) p508-14	1-27
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X	Dialog Information Services, File 5, BIOSIS PREVIEWS(R), Dialog accession no. 4582565, Biosis accession no. 28054884, Margaritis A et al: "Production and thermal stability characteristics of cellulase and xylanase enzymes from thielavia terrestris "; Scott, C.D.(ED), Biotechnology and Bioengineering Symposium, No. 13. 5th Symposium on Biotechnology for fuels and chemicals; Gatlinburg, Tenn., USA, May 10-13, 1983, VIII+672P	1-27
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X	Dialog Information Services, BIOSIS, Dialog accession no. 6602299, Biosis accession no. 86068850, Merchant P, et al: "Production of xylanase by the thermophilic fungus Thillavia terrestris"; & Biotechnol. Lett 10(7), 1988	1-27
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00033

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 5, BIOSIS PREVIEWS(R), Dialog accession no. 11585087, Biosis accession no. 98185087, Kvesitadze E G et al: "Isolation and Properties of a Thermostable Endoglucanase from a Thermophilic Mutant Strain of Thielavia terrestris"; & Applied Biochemistry and Biotechnology 50 (2). 1995. 137-143	1-27
A	Dialog Information Services, File 5, BIOSIS PREVIEWS(R), Dialog accession no. 9042524, Biosis no. 93027524, Gilbert M et al: "Characterization of the enzymes present in the cellulase system of thielavia-terrestris 255B"; & Bioresour Technol 39 (2). 1992. 147-154	1-27
A	WO 9523514 A1 (NOVO NORDISK A/S), 8 Sept 1995 (08.09.95), page 4, line 27 - line 30	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/04/97

International application No.
PCT/DK 97/00033

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9523514 A1	08/09/95	AU 1755095 A	18/09/95
		CA 2184591 A	08/09/95
		EP 0746206 A	11/12/96